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FOREWORD

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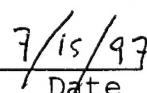
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INTRODUCTION

The Rak tyrosine kinase was originally identified in a PCR-based screen of protein kinases expressed in a breast cancer cell line and in a primary breast tumor (1). Rak is related to the Src tyrosine kinase and was expressed in approximately one-third of a series of primary breast tumors. Furthermore, the expression of Rak was elevated in epithelial cell lines and tissues, and was minimally detectable in non-epithelial cells, both at the RNA and protein level (1,2). Cloning of the full-length Rak cDNA revealed that Rak is approximately 50% homologous to members of the Src family and contains many of the structural features of these genes, including single SH2 and SH3 (Src homology 2 and 3, respectively) domains and a regulatory tyrosine at the carboxy terminus (2).

However, the structure of Rak was unique in that it contained a putative bipartite nuclear localization sequence spanning amino acids 168-181, and immunofluorescence and cell fractionation studies revealed that Rak localized to the nucleus. In addition, the localization of Rak varied between cell lines, being almost entirely nuclear in some cell lines (such as COS7 cells) and in the nucleus of only one third of BT20 breast cancer cells. Furthermore, the differential localization of Rak was detected in human tumors by immunohistochemistry. Therefore, one potential means of regulation for Rak is through a shift in its localization.

Based on the homology of Rak to Src-related kinases, we had originally anticipated that Rak would be a transforming gene. However, expression of Rak in a variety of cell lines causes growth inhibition (3). Furthermore, the expression of Rak from a dexamethasone-inducible promoter in NIH 3T3 cells causes a flat-cell morphology, which was consistent with the inhibition of growth. When Rak was transiently expressed in U2OS osteosarcoma cells and analyzed by flow cytometry, the cells arrested in G2/M (Craven, R.J. and Liu, E.T., unpublished data). Consistent with this, we found that Rak expression in epithelial cells decreases in the G2/M phase of the cell cycle (3). This suggested a model in which Rak expression normally drops at the G2/M phase of the cell cycle, allowing cell cycle progression. When Rak is expressed from an exogenous promoter, this interferes with its normal expression pattern, causing growth arrest.

We then searched for potential effectors of Rak's growth inhibitory activity. Using an *in vitro* protein interaction screen, we found that Rak binds to the cell cycle regulatory proteins Rb (the Retinoblastoma tumor suppressor protein, ref. 3) and Cdc2 (the Cell division control protein kinase, Craven, R.J. and Liu, E.T., unpublished data). Both appeared to be excellent candidates for effectors of Rak's growth inhibitory activity, and I have begun to analyze their contributions in this regard. Furthermore, a peptide sequence within the SH3 domain of Rak appeared to inhibit the kinase activity of Cdc2 in initial experiments, and I characterized this activity further. The three specific aims of this fellowship were as follows: 1) Determine the biological consequences of Rak localization, 2) Characterize the importance of the association between Rak and Rb and Cdc2, and 3) characterize the Rak-based Cdc2 inhibitory peptide.

Because of the progress described in the original grant application and the subsequent projects, I have satisfied the requirements for graduation and defended my thesis last fall. On November 9, 1996, I notified Dr. Isabelle Crawford that my graduation was imminent, and requested to stop receiving funding for this grant at the end of December 1996. As a result, this report will describe the work that I did from July 1, 1996- December 31, 1996, when my work on this grant ended. I am deeply grateful for the Army for the opportunity to write a grant and use the support for the completion of my graduate work. Simply writing the grant was a profound learning experience, and I look forward to writing more in the future.

BODY

Results and discussion

The purpose of Specific Aim 1 was to determine the biological consequences of Rak localization. We had previously found that Rak is a nuclear protein by immunofluorescence and sub-cellular fractionation (2). Furthermore, Rak contained a putative nuclear localization sequence (NLS) within its SH2 domain. To test whether this was indeed a nuclear localization signal, I mutated two of the amino acids from Arg-Arg-Arg to Gln-Arg-Gln and expressed this mutated form of Rak (called Rak-NLS-QRQ) in COS-7 cells. As expected, the wild-type Rak protein localized to the nucleus in this expression system, but the NLS mutant now fractionated to the cytoplasm. These experiments demonstrated that the basic sequence in the Rak SH2 domain plays a major role in the targeting of Rak to the nucleus.

We then wished to determine if this mutated form of Rak was biologically active. I had previously found that Rak inhibited the growth of mesenchymal calls by a colony formation assay (3), and performed the same type of analysis in the breast cancer cell line MCF10. The growth of these cells was not substantially inhibited by Rak, in keeping with the fact that they express moderate levels of Rak. However, when the Rak-NLS-QRQ mutant was expressed, the cells rapidly died. To determine the nature of this phenotype, I examined the DNA content of the cells by flow cytometry and found that the cells appeared to have undergone necrosis. Because this result was so unexpected, we decided that a complete characterization of this activity fell outside the scope of my graduate work.

In continuing these experiments, I think that it will be important to differentiate between two possible models for novel phenotypes in NLS mutants. Because the Rak NLS lies within the SH2 domain of the protein, mutations to this region may have multiple effects. For instance, it is possible that Rak normally inhibits growth by binding proteins through its SH2 domain. If the NLS mutant interferes with this binding, complex phenotypes may become detectable that would be difficult to explain in the context of localization.

The second specific aim was to characterize the biological importance of the interactions between Rak and Rb and/or Cdc2. I addressed the significance of Rak's binding to Rb by expressing Rak in cells

that were Rb+ or Rb-, selecting the U2OS and Saos-2 osteosarcoma cell lines, respectively. Our hypothesis was that if Rak required Rb for growth inhibition, then Rak would not inhibit growth in the Rb-minus cell line Saos-2. Indeed, Rak inhibited the growth of both cell lines by approximately 60%, and emergent colonies did not express Rak to detectable levels. Thus, we concluded that Rak does not require Rb for growth inhibition.

However, there are some important caveats to this conclusion. Saos-2 cells express the Rb-related proteins p107 and p130, and an interaction between Rak and these proteins may contribute to growth inhibition. Second, we have not analyzed the mechanism of Rak-mediated growth inhibition in different cell types such as mesenchymally-derived sarcoma cells. Because Rak is expressed primarily in epithelial cells, the potential contribution of Rb to Rak-mediated growth inhibition of epithelial cells was not addressed by these experiments. However, it does appear that Rb itself is not required for Rak's growth inhibition of osteosarcoma cells.

I attempted to assess the dependence on Cdc2 for the biological activity of Rak. Because Cdc2 is an essential gene, it was not possible to prepare cell lines which lack Cdc2. However, we hypothesized that if Cdc2 could be overexpressed, this might overwhelm the ability of Rak to interrupt the function of Cdc2. If Rak was dependent on Cdc2 for its function, Rak might no longer be able to inhibit growth when Cdc2 was overexpressed. Unfortunately, overexpression of Cdc2 by itself in MCF10 breast cancer cells caused them to die by an unknown mechanism. It is possible that excess Cdc2 might drive the cell into a premature mitosis, or by competition with other proteins for regulatory factors. In any event, the inability to express Cdc2 prevented us from performing this experiment. It is possible that other cell types may be less sensitive to Cdc2 overexpression, and might be a better system for understanding the contribution of Cdc2 binding to Rak's growth inhibitory activity.

The third specific aim was to analyze the ability of a peptide from the Rak SH3 domain to inhibit Cdc2. We had previously found that Rak contained an 11 amino acid insert in comparison to other related SH3 domains (2). The sequence, KRRDGSSQQLQ, did not share a high degree of homology with other known proteins. We had found that the Rak SH3 domain was capable of binding to Cdc2 in an *in vitro* binding assay, and that this domain could also inhibit the activity of Cdc2. Because other related SH3 domains did not inhibit Cdc2, we speculated that this sequence may contribute to the inhibition of Cdc2 by the Rak SH3 domain. Surprisingly, an 11 amino acid peptide spanning this insert was capable of inhibiting Cdc2. However, the inhibition was weak and only in the 100-500 uM range.

To further characterize this inhibitory activity, we synthesized shorter peptides containing portions of the original sequence, i.e. KRRD or QQLQ. I immunoprecipitated Cdc2 from a BT20 breast cancer cell lysate and performed an *in vitro* kinase assay using Histone H1 as a substrate. To test the effects of Rak-based peptides, varying concentrations of peptide were preincubated with the immunoprecipitated Cdc2 on ice for 20 min. before adding 32P- γ ATP to start the reaction. These KRRD or QQLQ sequences did not inhibit the activity of Cdc2 to a detectable degree. We then synthesized a new preparation of the original 11 amino acid peptide KRRDGSSQQLQ, and were not able to repeat our original growth inhibition

observation. We conclude that the original preparation of the 11 amino acid peptide may have contained a contaminant that inhibited the activity of Cdc2. Dr. Liu's lab is continuing to characterize the interaction between Rak and Cdc2 in the hope that other Cdc2 inhibitory sequences might be identified.

Experimental methods and procedures

For localization studies, Rak was expressed in COS-7 cells using the vector pCMV4-RakNT, encoding the amino terminal 200 amino acids of Rak fused in frame with the Flag epitope tag sequence. The Rak coding sequence was mutated by PCR-based mutagenesis. Rak-expressing plasmids were transiently transfected into COS-7 cells using the Lipofectamine reagent (Gibco BRL) and fractionated by lysis in 10 mM HEPES, 60 mM KCl, 1 mM EDTA, protease inhibitors, and 0.15% Triton X-100 and centrifugation, in which the supernatant contained the cytoplasmic proteins. The nucleus was then lysed in the same buffer containing 0.5% Triton X-100. Cellular fractions were analyzed by western blot and probed with the anti-Flag M2 monoclonal antibody.

For other transfection experiments, Rak was expressed in MCF10 breast cancer cells, U2OS and Saos-2 osteosarcoma cells using the pcDNA3-Rak vector, in which Rak was expressed from the cytomegalovirus promoter in the vector pcDNA3. Transfections were performed with the Lipofectamine reagent as described by the manufacturer and transfected clones were selected in neomycin at a concentration of 400 ug/ml. All of the cell lines were obtained from the American Type Culture Collection. Growth inhibition was calculated by staining the plates with crystal violet and counting the number of emerging colonies. The percent inhibition of growth was the number of colonies emerging following transfection with Rak divided by the number of colonies emerging following transfection with the host plasmid times 100.

For Cdc2 kinase assays, Cdc2 was immunoprecipitated from cell lysates in a lysis buffer containing 1% Nonidet P-40 using the Ab(17) anti-Cdc2 antibody from Santa Cruz. The pellet was washed three times with lysis buffer and once with kinase buffer (10 mM HEPES, pH 7.4, 5 mM MgCl₂, and 1 mM Dithiothreitol). The pellet was then resuspended in 25 ul kinase buffer. Where appropriate, 100-1000 uM peptide was added to the precipitated Cdc2 and incubated on ice for a further 30 min. The reaction was started by adding 1 ug of Histone H1 (Boehringer Mannheim) and 10 uCi 32P-gATP, and was incubated at 30oC for 20 min. Reactions were then analyzed by SDS-PAGE and autoradiography.

Recommendations

In general, I believe that two experiments in Dr. Liu's lab will reveal a great deal about the function of Rak in vivo. First, Dr. Liu is continuing to study the phenotypes of a Rak mutant containing point mutations in the nuclear localization signal. Second, Dr. Liu is preparing an inducible system that will be more workable than the dexamethasone-inducible system that I prepared. Together, these experiments

will offer a more refined analysis of the effects of overexpressing Rak in tumor cells and understanding the contribution of the nuclear localization of Rak.

CONCLUSIONS

Rak is a tyrosine kinase that was originally identified in breast cancer cells. We have found that Rak is related to the Src family of tyrosine kinases, but is localized to the nucleus and has growth inhibitory activity. Furthermore, Rak associates with the cell cycle regulatory proteins Rb and Cdc2. These experiments indicate that binding to Rb is not an essential part of the mechanism through which Rak inhibits cell growth. The localization of Rak to the nucleus appears to be an important part of its regulation, and a mutant of Rak which localized to the cytoplasm caused a novel phenotype for a tyrosine kinase by triggering necrosis in a breast cancer cell line. Thus, Rak is a growth inhibitory tyrosine kinase with a complex regulation that may play an important role in growth regulation in epithelial cells.

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